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Growing Membranes *In Vitro* by Continuous Phospholipid Biosynthesis from Free Fatty Acids

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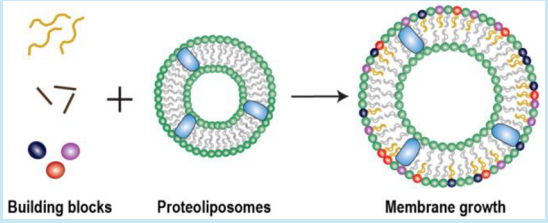
Supporting Information

ABSTRACT: One of the key aspects that defines a cell as a living entity is its ability to self-reproduce. In this process, membrane biogenesis is an essential element. Here, we developed an *in vitro* phospholipid biosynthesis pathway based on a cascade of eight enzymes, starting from simple fatty acid building blocks and glycerol 3-phosphate. The reconstituted system yields multiple phospholipid species that vary in acyl-chain and polar headgroup compositions. Due to the high fidelity and versatility, complete conversion of the fatty acid substrates into multiple phospholipid species is achieved simultaneously, leading to membrane expansion as a first step toward a synthetic minimal cell.



KEYWORDS: *phospholipid biosynthesis, membranes, synthetic cell, enzymatic conversion, membrane proteins*

The construction of a synthetic minimal cell is a main challenge in synthetic biology, where bottom-up approaches are being utilized to create biological mimicking structures. The idea behind building a synthetic minimal cell is to assemble a minimum of cellular components (nucleic acids, enzymes, lipids, *etc.*) to create a "living cell", capable of executing basal cellular functions.[1−5] One of the key features defining a cell as a living entity is its ability to self-reproduce in which the expansion of the cellular membrane is a critical aspect. One approach toward a growing boundary layer is the *de novo* synthesis of its building blocks followed by its self-assembly. Previous attempts on *de novo* synthesis of membranes have mainly been based on fatty acids (FAs), due to their ability to spontaneously assemble into micelles.[6−8] However, the intrinsic properties of FAs give them a dynamic profile, which allow them to appear as vesicles as well. Large multilamellar FA vesicles could be grown by feeding the system with FA micelles, causing the multilamellar structures to ultimately divide.[9] Moreover, a simple nonenzymatic FA synthesis reaction could be coupled to vesicular growth and division, as an example of autocatalytic self-reproduction.[10] Although, these are first steps toward the synthetic engineering of a self-reproducing system, membranes based on FAs are intrinsically unstable and do not encompass a sizable lumen. In contrast, biological membranes consist of much more complex molecules, of which phospholipids are the major component. Their amphipathic character allow them to orient in a bilayer-like structure to support the barrier function. Unlike the previously mentioned simple fatty acid containing membrane mimics, additional intrinsic properties of the different types of phospholipids are of major importance.[11,12] This is illustrated by the cytoplasmic membrane of *Escherichia coli*, which consists almost completely out of the zwitterionic phospholipid phosphatidylethanolamine (PE, 70−75%), the anionic phosphatidylglycerol (PG, 20−25%) and depending on the growth phase, varying amounts of cardiolipin (CL, 0−10%). While CL is a nonessential component, a proper balance is needed between the bilayer forming PG and the nonbilayer PE to support the activity of membrane proteins. Anionic phospholipids are needed to stabilize membrane proteins and promote membrane association of peripheral membrane proteins, while nonbilayer lipids are needed for membrane protein folding and assembly.[13,14]

Evidently, any synthetic biology approach toward a self-reproducing membrane must rely on phospholipids. Although some successes have been obtained for *de novo* vesicle formation by assembly and chemical synthesis of phospholipid-like structures,[15−18] most research has focused on the addition of components to a pre-existing membrane. Several attempts have been made to mimic boundary layer growth *in vitro* using synthetic compartments, of which liposomes are considered the most suitable model due to their structural similarity with cellular membranes. For example, chemical synthesis of artificial phospholipid resulted in liposomal growth and subsequent division of the giant vesicles, which could be coupled to amplified DNA proliferation.[19,20] A more biology based approach is illustrated by the enzymatic addition of FAs to pre-existing liposomes resulting in spontaneous incorporation, followed by expansion of the membrane.[21] Further enzymatic conversion of fatty acids into phospholipids is,

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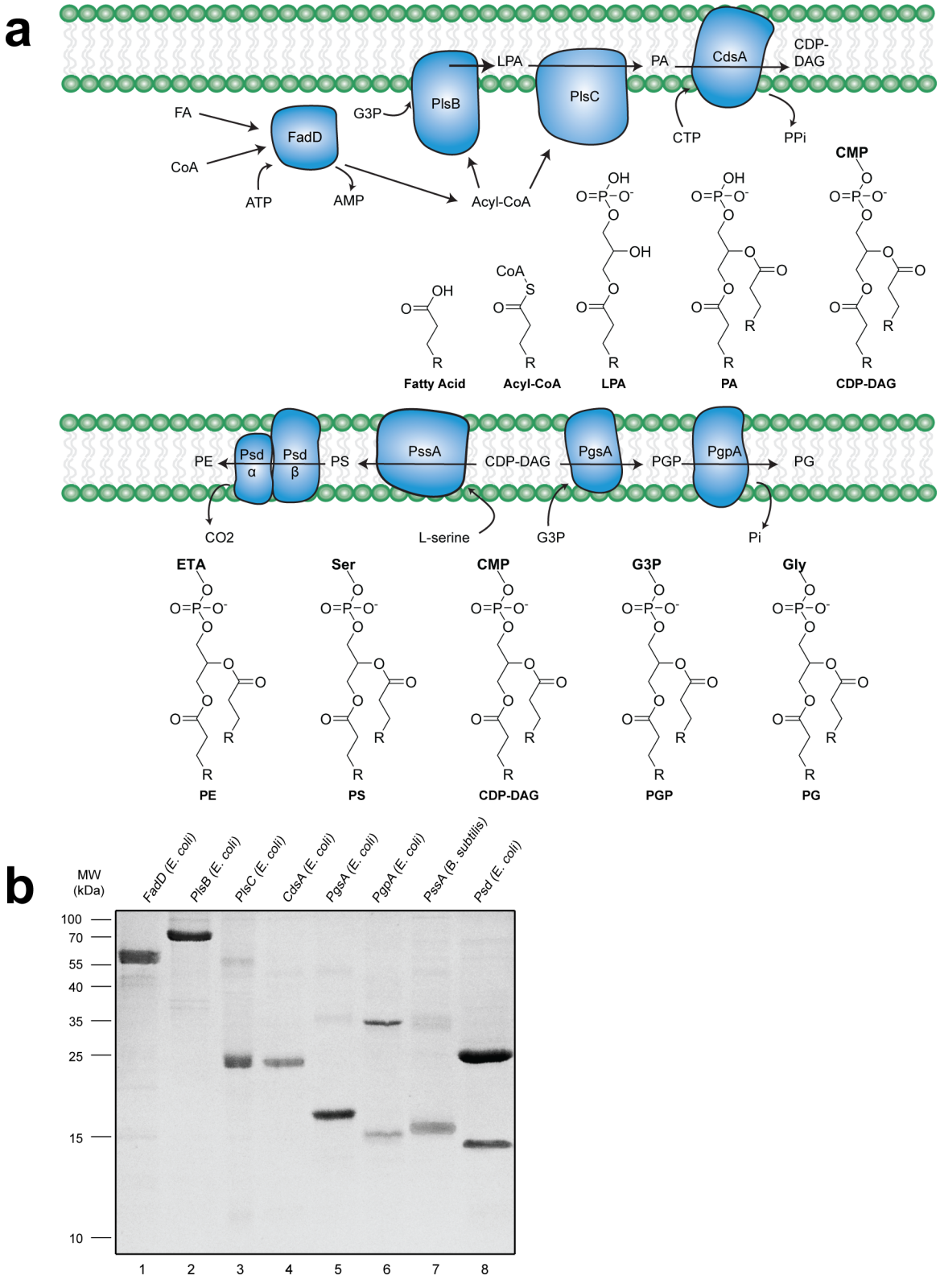

Figure 1. Schematic representation of the *in vitro* phospholipid biosynthesis pathway and protein purification. (a) FadD converts simple fatty acid (FA) building blocks into acyl-chain donors which are utilized by the enzymes PlsB and PlsC to form lysophosphatidic acid (LPA) and phosphatidic acid (PA), respectively. PA is further converted into CDP- diacylglycerol which serves as precursor for biosynthesis of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). (b) Coomassie stained SDS-PAGE gel of the eight lipid biosynthesis enzymes purified by Ni-NTA chromatography. The upper-band in lane 6 displays a dimer of PgpA. The two bands in lane 8 represent the two individual subunits of the Psd enzyme.

however, essential to support the activity of membrane proteins and to obtain a stable membrane system.

The most realistic representation of membrane growth would be to perform the synthesis from within a liposome. In

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this respect, enzymes derived from an *in vitro* transcription/translation system have been encapsulated into liposomes[22] and enzymatic activity was confirmed, Unfortunately, the low fidelity of this system combined with experimental limitations of liposome encapsulation, render phospholipid biosynthesis highly inefficient and insufficient to achieve appreciable liposome growth. To avoid the problem of low enzyme production yields, liposomes were recently fed with newly synthesized proteins and lipid precursors from the outside.[23] However, also in this case phospholipid formation was inefficient and the claimed membrane growth of <1% from supplied FA-CoA was too small to experimentally demonstrate expansion. Furthermore, FA-CoA is an expensive compound and therefore an unsuitable building block for the construction of an economically viable synthetic cell. Therefore, any approach aiming at appreciable membrane growth a mechanism of CoA recycling is needed.

To circumvent issues with protein synthesis through transcription/translation, purified enzymes can be used. We have recently pioneered this method to resolve some of the remaining open questions in archaeal ether phospholipid biosynthesis.[24] Complete reconstitution allows for a simplified design with the potential to produce substantial amounts of phospholipids to generate a membrane that grows by expansion. Such a system would allow for studies on complex processes that in living cells are linked to membrane expansion, among which cell division and the insertion of membrane proteins into the lipid bilayer. Here, we report on the design and engineering of a complete *in vitro* phospholipid biosynthesis pathway using eight purified (membrane) proteins, to realize the enzymatic conversion of simple fatty acid precursors into the final phospholipid species phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), two major components of bacterial membranes. Since synthesis coincides with the incorporation of lipid precursors into a pre-existing liposomal membrane, biosynthesis of chemical amounts of phospholipids resulted in membrane expansion and yielded membranes in which the polar headgroup and acyl chain composition can be altered on demand.

RESULTS AND DISCUSSION

Phospholipid Biosynthesis Pathway Design. Phospholipid synthesis has been studied in great detail in the bacterium *Escherichia coli* and the enzymes involved have been identified and characterized.[12,25,26] We used the *E. coli* system as a template to develop a versatile *in vitro* phospholipid biosynthesis pathway, combined with enzymes derived from other sources (Figure 1a). The main principle of this pathway is based on a feed with free fatty acids (FAs) to yield fatty acyl-Coenzyme A (FA-CoA) that is utilized with glycerol 3-phosphate (G3P) to generate phosphatidic acid (PA). The PA is further converted into phospholipids with different head groups and acyl chain composition through a series of additional enzymatic steps. In *E. coli* phospholipid synthesis starts with the biosynthesis of FA-CoA by the type II fatty acid synthase (FASII) using malonyl-CoA and acyl-CoA as substrates. However, a more simple approach is to use the catabolic enzyme FadD involved in β-oxidation of FAs.[27,28] Whereas, FASII is a multicomplex enzyme, that carries out cycled chain-elongation and subsequent acyl-carrier protein coupling, FadD uses already premade FAs in a simple two-step conversion, resulting in the direct production of FA-CoA from FAs, adenosine-triphosphate (ATP) and CoA. The use of FadD further adds to the versatility of the system, as the acyl chain composition in the system can be readily determined by the feed of different FAs. The next step is the attachment of two acyl-chains to glycerol 3-phosphate (G3P). In *E. coli*, two membrane-associated proteins are responsible for these acylation reactions, *i.e.*, glycerol 3-phosphate acyltransferase (PlsB) and lysophosphatidic acid acyltransferase (PlsC).[26,29] PlsB transfers an acyl-chain on to the 1-position of G3P, resulting in lysophosphatidic acid (LPA). Subsequently, PlsC attaches another acyl-chain to the 2-position of LPA, resulting in PA, a central intermediate in phospholipid biosynthesis. As both these enzymes recognize FA-CoA as an acyl-chain donor, they can be easily combined with the aforementioned FadD. Moreover, the CoA is completely regenerated in these steps and thus made available to FadD to activate further FAs. Although a PA-only membrane may fulfill some basic requirements such as the barrier function, it will not support the activity of membrane proteins. Therefore, PA needs to be converted into the two key phospholipid species, phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). For this purpose, the central precursor cytidine diphosphate-diacylglycerol (CDP-DAG) needs to be synthesized from PA with cytidine triphosphate (CTP) by CDP-diacylglycerol synthase (CdsA). Next, two additional conversions are needed to yield either PE or PG. CDP-DAG can be converted into phosphatidylserine (PS) by PS synthase (PssA) utilizing serine, whereupon PS is converted into PE by PS decarboxylase (Psd). CDP-DAG can also be converted into phosphatidylglycerol-3-phosphate (PGP) by PGP synthase (PgsA) followed by the removal of the 3-phosphate by one of the PGP phosphatases (PgpA, PgpB, or PgpC) yielding PG.[12,25,26] We have previously demonstrated the *in vitro* conversion of PA into PG and PS by using the above-mentioned enzymes from *E. coli*.[24] Interestingly, the enzyme PssA from *E. coli* was far less active *in vitro* compared to the corresponding enzyme from *Bacillus subtilis*. Therefore, to develop a high fidelity *in vitro* phospholipid synthesis pathway, the *B. subtilis* PssA was employed.

To construct an *in vitro* phospholipid biosynthesis pathway, a substantial quantity of the individual enzyme is needed. Herein, the genes of all required enzymes, *i.e.*, 8 in total (Table 1), were cloned separately into a His-tag containing pET-based overexpression vector allowing their overproduction. As most of the enzymes are membrane (associated) proteins, membranes were isolated from the overproducing strains, after

Table 1. Proteins Used in This Study and Their Characteristics

protein	molecular mass (kDa)	number of (predicted) TM segments	protein type
FadD	62	0	Cyt[a]
PlsB	91	0	PMP[b]
PlsC	27	1	PMP
CdsA	31	9	IMP[c]
PssA	18	5	IMP
Psd (subunit α)	14	0	PMP
Psd (subunit β)	29		
PgsA	21	4	IMP
PgpA	19	3	IMP

[a]Cyt, cytosolic. [b]PMP, peripheral membrane protein. [c]IMP, integral membrane protein.

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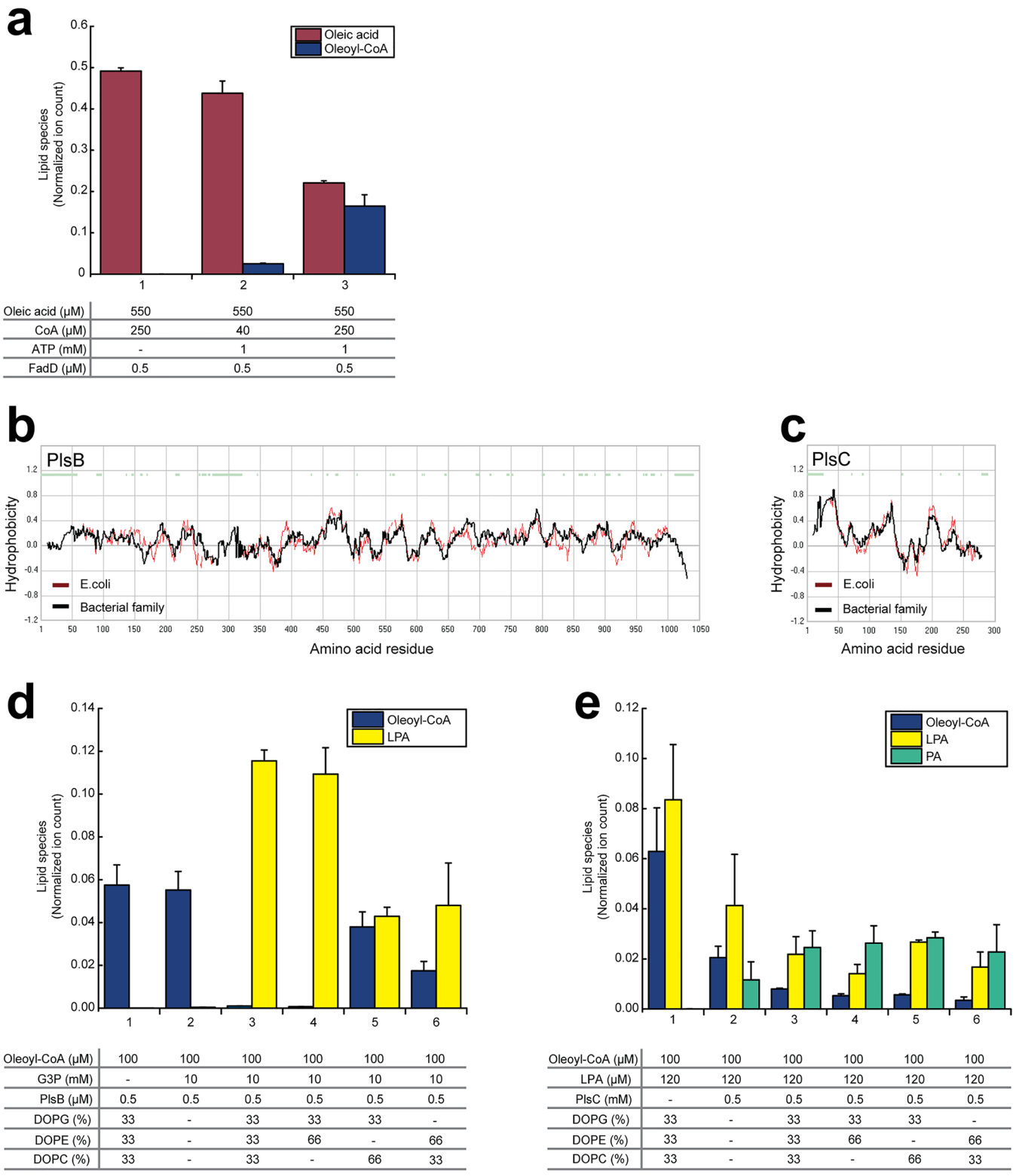

Oleic acid (μM)	550	550	550
CoA (μM)	250	40	250
ATP (mM)	-	1	1
FadD (μM)	0.5	0.5	0.5

Oleoyl-CoA (μM)	100	100	100	100	100	100
G3P (mM)	-	10	10	10	10	10
PlsB (μM)	0.5	0.5	0.5	0.5	0.5	0.5
DOPG (%)	33	-	33	33	33	-
DOPE (%)	33	-	33	66	-	66
DOPC (%)	33	-	33	-	66	33

Oleoyl-CoA (μM)	100	100	100	100	100	100
LPA (μM)	120	120	120	120	120	120
PlsC (mM)	-	0.5	0.5	0.5	0.5	0.5
DOPG (%)	33	-	33	33	33	-
DOPE (%)	33	-	33	66	-	66
DOPC (%)	33	-	33	-	66	33

Figure 2. *In vitro* activity of purified FadD, PlsB and PlsC. (a) FadD activity: reactions were performed in the presence of purified FadD, as specified. Subsequently, 0.1% of the internal standard DDM was added. Products were analyzed by LC−MS, normalized for the internal standard and plotted on the *y*-axis. Hydropathy profile alignment of (b) *E. coli* PlsB and (c) PlsC (red line), with the averaged hydropathy profile of their bacterial protein family (black line). (d) PlsB and PlsC activity. Reactions were performed in the presence of purified enzyme and 0.1% DDM. Products were analyzed by LC−MS, normalized and plotted.

which the enzymes were solubilized with the detergent *n*-dodecyl-*β*-D-maltoside (2%, w/v). Subsequently, proteins were isolated using Ni-NTA agarose affinity chromatography, resulting in pure enzyme fractions (Figure 1b).

Formation of Phosphatidic Acid in Detergent Solution. For the biosynthesis of the bilayer forming phospholipid PA, only three enzymes are needed (Figure 1a). FadD catalyzes the coupling of CoA to a fatty acid substrate.

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This two-step process is driven by the hydrolysis of ATP into AMP, resulting in the production of the FA-CoA.[30] When purified FadD was incubated with ATP, CoA and oleic acid (C18:1), mass spectrometry demonstrated the formation of the CoA derivative (Figure 2a) concomitantly with the consumption of the FA. In the absence of ATP, no conversion was observed. CoA-limiting conditions resulted in a proportional consumption of oleic acid and generation of oleoyl-CoA, and increased CoA concentrations resulted in higher levels of oleoyl-CoA (Figure 2a), without any product feedback inhibition (Figure S1A). The activity of FadD was not further influenced by the presence of phospholipids (Figure S1B) and could be carried out in solution. These data demonstrate that FadD can be used to supply the anticipated biosynthesis system with FA-CoA derivatives.

PlsB and PlsC both utilize FA-CoA to attach the acyl chain on the first and second position of glycerol-3-phosphate (G3P), respectively. Previous studies on overexpressed PlsB suggest that the enzyme is fully integrated into the membrane.[31] However, an analysis of the average predicted hydropathy-profile of a family of bacterial PlsB homologues (Figure 2b), does not signify any transmembrane segment, suggesting the enzyme may rather be membrane associated. Nevertheless, PlsB could be purified from membranes, and efficiently produced LPA from oleoyl-CoA and G3P, but only in the presence of phospholipids (mixture of DOPG, DOPE and DOPC, 1:1:1 molar ratio) (Figure 2d). Interestingly, the oleoyl-CoA was completely converted into LPA, which contradicts an earlier study that suggested a negative feedback inhibition by free CoA on PlsB activity.[23] Although a phospholipid requirement was noted before,[22,32] the need for individual phospholipid species is as yet unknown. To further address this, PlsB activity assays were performed in the presence of liposomes with different phospholipid compositions. Whereas in the presence of both DOPE and DOPG, complete conversion of oleoyl-CoA into LPA was obtained in 30 min, replacement of either phospholipid species for DOPC resulted in significantly lower conversion efficiencies.

In contrast to PlsB, the average hydropathy analysis of a family of bacterial PlsC homologues (Figure 2c) predicts a single transmembrane segment at the N-terminus. This region may function as a membrane anchor to catalyze LPA to PA conversion at the membrane interface. PlsC was isolated from membranes of an *E. coli* overexpressing strain (Figure 1b). When purified PlsC in detergent solution was fed with oleoyl-CoA and LPA, formation of PA was observed (Figure 2e). Although the addition of phospholipids seems to enhance the PA production, they are not essential for PlsC its activity (Figure 2e). When PlsC was combined with PlsB in detergent solution, a feed with oleoyl-CoA and G3P resulted in the formation of PA (Figure S2), thereby setting the basic requirements for the projected phospholipid biosynthesis pathway.

Two fundamentally different approaches toward a growing membrane can be perceived, *i.e.*, the *de novo* synthesis of membranes through self-assembly of the phospholipids into a bilayer, and growth of an already existing membrane through further addition of phospholipids. The *in vitro* phospholipid synthesis pathway did not support *de novo* membrane synthesis because of the necessity of phospholipids for PlsB mediated acyl-chain attachment. Moreover, many of the other proteins involved in phospholipid synthesis are multimembrane spanning enzymes, which require membranes for proper functioning. Therefore, the system depends on the use of already pre-existing membranes for *in vitro* phospholipid synthesis and membrane expansion, thereby representing the natural situation.

Reconstitution of Phosphatidic Acid Formation. To establish a system for membrane growth, the enzymes needed for PA formation were combined in a single reaction setup and reconstituted into liposomes. Herein, PlsB and PlsC were added to a reaction mixture containing liposomes such that the final detergent concentration remained below its critical micelle concentration (CMC) (0.01%). In this way, the liposomes remain intact. To these membranes FadD was added and when supplied with oleic acid, CoA, ATP and G3P, a complete conversion of oleic acid into PA was observed (Figure 3a). When only FadD and PlsB were present, small amounts of PA were formed, likely because of a contamination of PlsB with some PlsC.

Using this liposomal system, the phospholipid requirement was re-examined. In the presence of both DOPG and DOPE, large quantities of oleic acid (2.7 mM) were completely converted into PA (Figure 3b). In the absence of DOPE, the yield of PA formation was reduced by 50% even though reactions were carried out overnight, whereas in the absence of PG, still complete conversion was obtained under the aforementioned conditions. Thus, in the liposomal system, PlsB depends more strongly on DOPE than on DOPG.

An important feature of the three-step cascade reaction toward PA biosynthesis is the recycling of CoA. Recycling was validated by testing PA formation at concentrations of CoA that were up to 65-fold lower than that of oleic acid, which thus would limit FadD for the formation of oleoyl-CoA. Indeed, in the presence of FadD and limiting amounts of CoA (50 μM), only part of supplied oleic acid (2.7 mM) was converted into oleoyl-CoA (Figure 3b). Further addition of PlsB and PlsC resulted in the conversion of all oleic acid into LPA/PA which implies that the synthesis involves recycling of CoA. Furthermore, when the amount of the PlsB and PlsC enzyme is taken into account, it can be concluded that the system not only recycles CoA but also catalyzes at least 2500 turnovers under the conditions employed. Following this procedure over time, in about 6 h almost all oleic acid is converted into PA (Figure S3).

Due to the broad substrate specificity of FadD in β-oxidation, the FadD dependent conversion of other FAs into their FA-CoA derivatives would provide a mechanism to generate phospholipids with an acyl chain composition that mimics that of biological membranes. In addition to oleic acid (C18:1). three other fatty acids, *i.e.*, stearic acid (C18:0), palmitoleic acid (C16:1) and palmitic acid (C16:0) were tested for PA biosynthesis. Herein, an equimolar mixture of these four fatty acids was supplied as substrates to FadD, and formation of the different LPA and PA species was examined by mass spectrometry. In the presence of FadD and PlsB, all FAs were converted *via* their respective FA-CoA derivatives into the corresponding LPA molecules (Figure 4a,b). Addition of PlsC results in the production of almost all possible variants of PA, although a preference is noted for species with acyl-chains that contain at least one double bond (Figure 4c). Furthermore, stearoyl-CoA and LPA (C18:0) both appear to be less preferred substrates as these were only slowly incorporated into PA (Figure 4b,c). Since this phenomenon was not apparent when only the combined activity of FadD and PlsB was tested, these data suggest that PlsC prefers the unsaturated and shorter acyl

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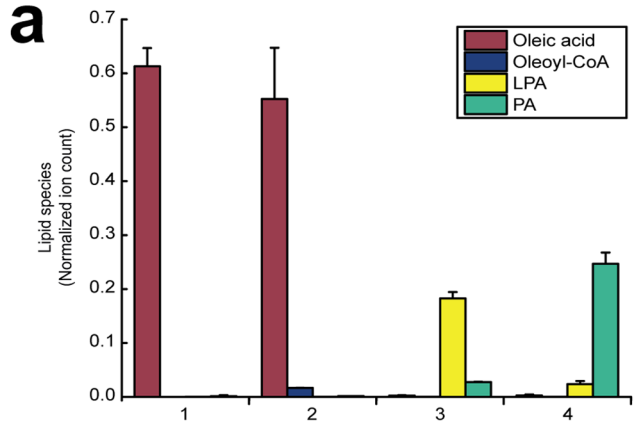
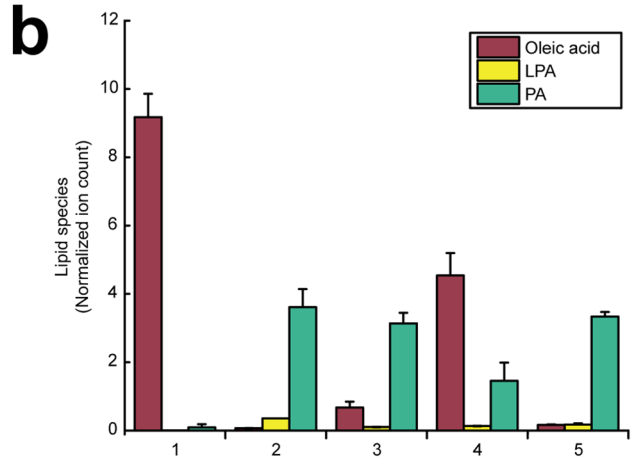


Oleic acid (μM)	550	550	550	550
CoA (μM)	50	50	50	50
ATP (mM)	-	1	1	1
FadD (μM)	0.5	0.5	0.5	0.5
G3P (mM)	-	-	10	10
PlsB (μM)	-	-	0.5	0.5
PlsC (μM)	-	-	-	1.5
Lipids (μM)	2,67	2,67	2,67	2,67



Oleic acid (μM)	2700	2700	2700	2700	2700
CoA (μM)	50	50	50	50	50
ATP (mM)	-	4	4	4	4
FadD (μM)	0.5	0.5	0.5	0.5	0.5
G3P (mM)	10	10	10	10	10
PlsB (μM)	0.5	0.5	0.5	0.5	0.5
PlsC (μM)	1.5	1.5	1.5	1.5	1.5
DOPG (%)	33	33	33	33	-
DOPE (%)	33	33	66	-	66
DOPC (%)	33	33	-	66	33

Figure 3. *In vitro* synthesis of PA by FadD, PlsB and PlsC. (a) Stepwise conversion of oleic acid into PA. Reactions were performed in the presence of purified enzymes reconstituted into liposomes. Products were analyzed by LC−MS, normalized for the internal standard POPG and plotted. (b) Synthesis of PA at high oleic acid concentration. Reactions were performed as described above, after which 0.1% of the internal standard DDM was added. Lipid products were analyzed as described above and plotted.

chain substrates. This matches the *in vivo* distribution of various acyl chains in the phospholipids of *E. coli*[33−35] Summarizing, the reconstituted system supports a three-step conversion of a multitude of FAs and G3P into PA with high efficiency, which is limited only by the amount of FAs supplied to the system.

Reconstitution of Polar Headgroup Attachment. The second stage of phospholipid biosynthesis is the formation of CDP-diacylglycerol (CDP-DAG), and the subsequent attachment of polar head groups. Previously, we have shown that PA can be converted *in vitro* into PG and PS with the corresponding enzymes in detergent solution.[24] However, for membrane growth these reactions need to be performed in the absence of detergent. As several of the enzymes involved in polar headgroup addition are integral membrane proteins (Table 1), reconstitution into liposomes was again achieved by low detergent incorporation keeping the concentration of *n*-dodecyl-*β*-D-maltopyranoside below the CMC.

Biosynthesis of PE and PG starts with the production of the central precursor CDP-DAG from PA and CTP by the enzyme CdsA, which attaches a CDP onto PA by dephosphorylating CTP. In the absence of CTP, the PA synthesized by the enzyme cascade FadD/PlsB/PlsC could not be converted by CdsA (Figure 5a,b). Addition of CTP resulted in formation of CDP-DAG, but the activity was low and most of the PA remained unused. Increasing the reaction time or the CdsA concentration did not improve biosynthesis (data not shown). We hypothesize that the limited PA conversion is caused by a feedback inhibition of CdsA by its product CDP-DAG, a phenomenon that has been observed for the CDP-DAG synthesizing enzyme from *M. smegmatis* as well.[36] Therefore, the reaction was coupled to the conversion of CDP-DAG into phosphatidylglycerol-3-phosphate (PGP). This reaction is catalyzed by the enzyme PgsA that utilizes the glycerol 3-phosphate (G3P) already present in the assay. Indeed, in the presence of PgsA, high level production of PGP was observed while the further addition of the phosphatase PgpA caused the complete conversion of PA into PG (Figure 5a). Similarly, a high level of PS production was observed when the system was supplemented with PssA and its substrate L-serine. Subsequent addition of phosphatidylserine decarboxylase, Psd, resulted in formation of PE (Figure 5b). In these experiments, introduction of the polar headgroup enzymes and their substrates resulted in a complete conversion of PA. Since CDP-DAG now is an intermediate in the reaction and does not accumulate to high levels, these data support the notion of feedback inhibition of CdsA by its product CDP-DAG. More importantly, the work demonstrates the functional reconstitution of the entire phospholipid biosynthesis pathway with purified enzymes reconstituted in liposomes.

Reconstitution of a Mixed Phospholipid Membrane. The next aim was to simultaneously synthesize PE and PG *in vitro*, by combining all the enzymes used in the aforementioned pathway thereby mimicking phospholipid biosynthesis in *E. coli* cells. In addition, the exact amounts of phospholipids produced were quantified by calibration of the mass spectrometry signals based on PE and PG standards (Supporting Information). Six enzymes involved in either PE or PG biosynthesis from FAs were mixed, and synthesis was initiated by the addition of 2.7 mM oleic acid. This resulted in the production of 1.2 mM PG (90%) and 1.35 mM PE (95−100%), respectively (Figure 5c), which is close to the theoretically expected conversion efficiency as two oleic acid units are needed to form a single phospholipid. Subsequently, all eight enzymes were mixed to yield a reaction in which both PE and PG are produced simultaneously. By varying the ratio of the PG forming enzymes PgsA and PgpA, and the PE forming enzymes PssA and Psd, the ratio of PG over PE could be adjusted such that a phospholipid mixture is obtained that mimics the typical native

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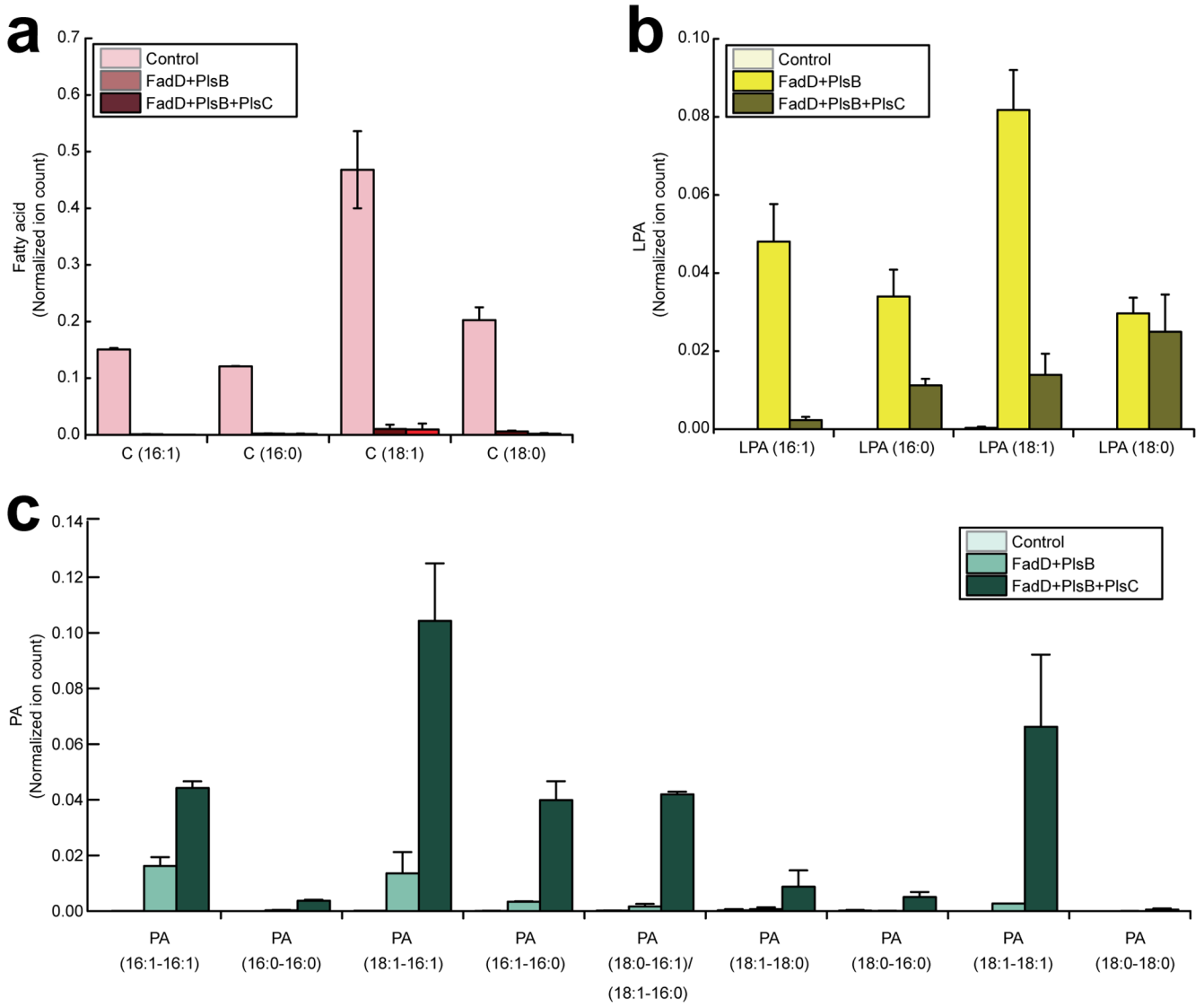

Figure 4. Synthesis of PA with varying acyl chain composition. An equimolar ratio of four different fatty acid species were mixed together and incubated with FadD, PlsB and with or without PlsC, and reconstituted into liposomes in the presence or absence (control) of ATP as indicated. Levels of the four fatty acid species (a), four LPA species (b) and nine PA species (c) are displayed for the three reaction conditions. Products were analyzed by LC−MS, normalized for the internal standard POPG and plotted.

molar ratio of 30:70 (Figure 5c). Again, in these reactions the conversion of FA into phospholipid is nearly complete. These data show that the lipid species composition of the membranes can be controlled on demand.

Although phospholipids are synthesized from within the cell, in our system substrates and enzymes are supplied from outside of the cell. Hereby, we avoid the problem of low substrate availability encountered in the past during attempts to mimic vesicle growth from within liposomes. Our approach differs dramatically from previous attempts to generate phospholipid *in vitro*[22,23] that could only yield minute quantities of phospholipids under poorly controlled conditions. As most of the proteins involved in phospholipid synthesis are membrane proteins, correct insertion/reconstitution is essential for their functionality. *In vivo* this is a highly regulated process where membrane insertion and folding occurs *via* the Sec-translocon in conjunction with protein synthesis at the ribosome. Earlier attempts to synthesize phospholipids were based on a low fidelity *in vitro* enzyme transcription/translation system, which resulted in slow and only partial (50%) conversion of supplied substrates, while product quantities were too low to observe any appreciable membrane growth. Instead, we used the membrane proteins isolated from overexpressing *E. coli* cells and these were reconstituted into pre-existing liposomes through detergent dilution. As a result, complete conversion of large amounts of substrate through all expected intermediates into multiple phospholipid species occurred simultaneously.

Membrane Expansion by Phospholipid Biosynthesis. To visualize membrane expansion, a fluorescent assay was used which is based on the self-quenching properties of the lipophilic fluorophore octadecyl rhodamine B (R18). This probe is commonly used to follow membrane mixing during vesicle fusion, and the degree of fluorescence quenching decreases upon reduction of the R18 concentration in the membranes.[37] Thus, phospholipid biosynthesis should result in increased levels of R18 fluorescence. The pre-existing liposomes were supplemented with 5 mol % R18, resulting in highly quenched

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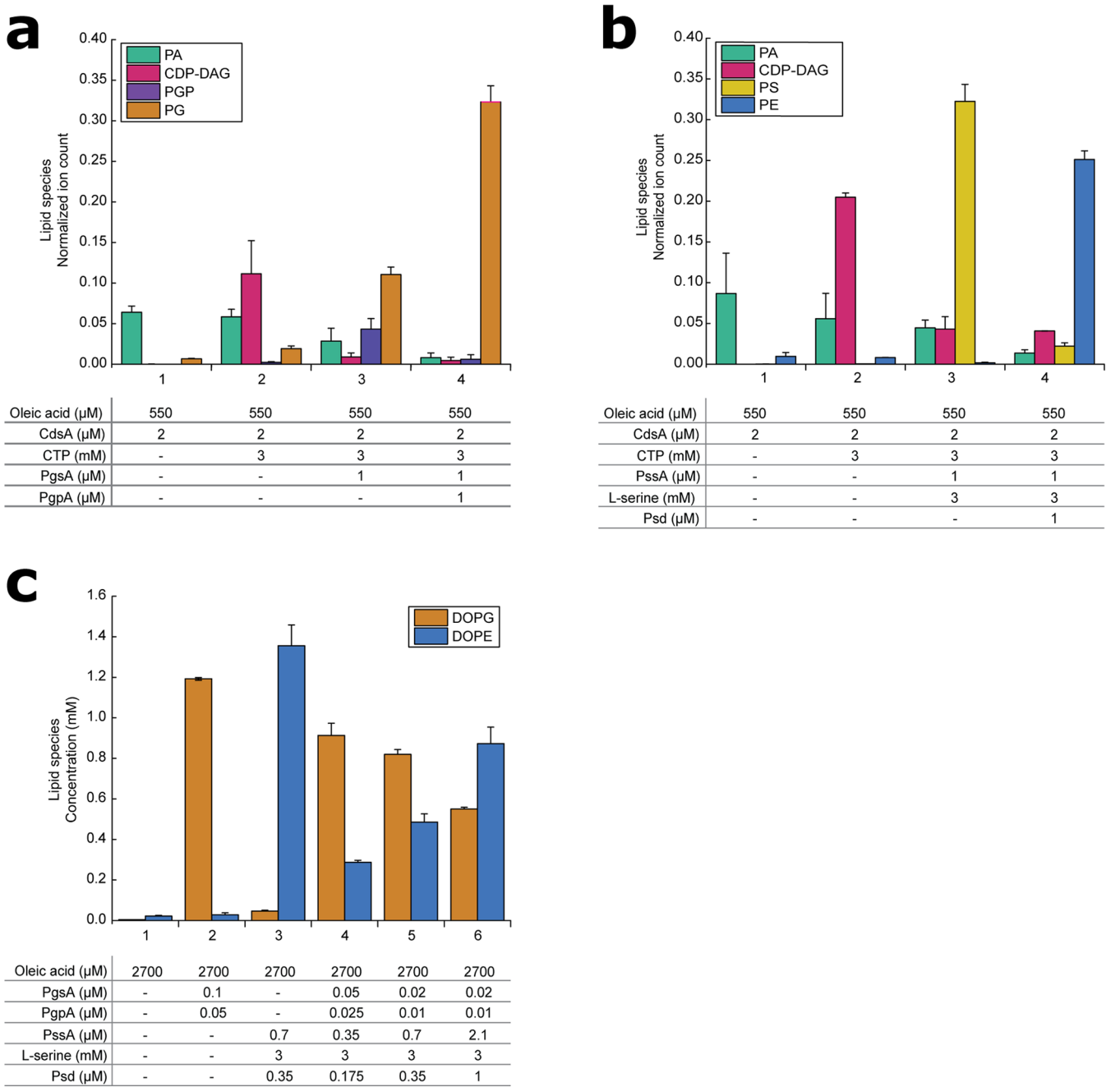

Oleic acid (μM)	550	550	550	550
CdsA (μM)	2	2	2	2
CTP (mM)	-	3	3	3
PgsA (μM)	-	-	1	1
PgpA (μM)	-	-	-	1

Oleic acid (μM)	550	550	550	550
CdsA (μM)	2	2	2	2
CTP (mM)	-	3	3	3
PssA (μM)	-	-	1	1
L-serine (mM)	-	-	3	3
Psd (μM)	-	-	-	1

Oleic acid (μM)	2700	2700	2700	2700	2700	2700
PgsA (μM)	-	0.1	-	0.05	0.02	0.02
PgpA (μM)	-	0.05	-	0.025	0.01	0.01
PssA (μM)	-	-	0.7	0.35	0.7	2.1
L-serine (mM)	-	-	3	3	3	3
Psd (μM)	-	-	0.35	0.175	0.35	1

Figure 5. *In vitro* biosynthesis of (a) PG, (b) PE and (c) mixtures of PE and PG from oleic acid and glycerol 3-phosphate. Reactions were performed in the presence of purified enzymes reconstituted into liposomes. Products were analyzed by LC−MS, normalized for the internal standard POPG and plotted.

fluorescence, which was relieved after the membranes were solubilized with the detergent Triton X-100. Subsequently, the enzymes and their substrates where added to the R18 labeled liposomes to initiate phospholipid synthesis. This resulted in an immediate increase of the R18 fluorescence that, by the addition of Triton X-100, increased to the reference maximal fluorescence level (Figure 6). The sudden increase in fluorescence, did not dependent on phospholipid biosynthesis, but can be solely attributed to the addition of FAs causing swelling and expansion of the liposomes. Remarkably, the conversion of oleic acid into PA by the added enzymes and their substrates did not result in a further change in fluorescence, although under these same conditions mass spectrometry demonstrates that all FAs were converted into PA (Figure 3b). The data suggest that oleic acid initially partitions into the liposomes, causing membrane expansion. However, this expansion is maintained upon the complete conversion of the FAs into PA. Since the level of R18 quenching is linearly correlated with the concentration of R18 in the membrane, the degree of membrane expansion can be calculated being 25−30%. Based on the amount of FAs provided (2.7 mM), and phospholipid present in the system (2.67 mM), the maximum expansion expected upon complete conversion of FAs into PA is 30%, which coincides with the experimentally obtained value. The membrane expansion in this system is only limited by depletion of substrate.

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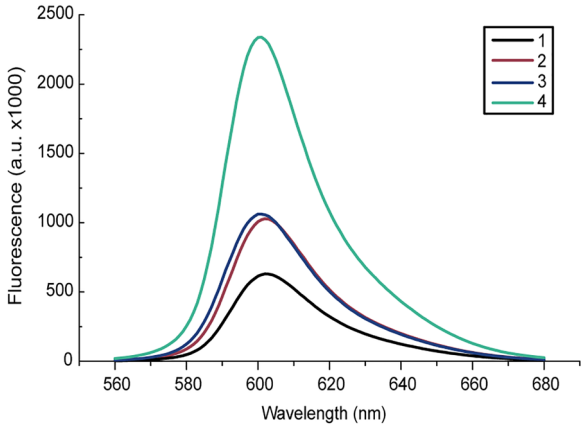

Figure 6. Phospholipid biosynthesis induced membrane expansion of liposomes. Membrane expansion was measured with the R18-self-quenching assay. Fluorescent emission spectra of R18 containing liposomes excited at 540 nm were recorded for the liposomes before (trace 1) and after (trace 2) the addition of 2.7 mM oleic acid and the complete conversion of oleic acid into PA (trace 3) by FadD, PlsB and PlsC as described in the legends of Figure 3b. Addition of Triton X-100 results in maximum fluorescent levels (trace 4) and was used to normalize the samples.

We noted that during the process of phospholipid production (PA, PE and/or PG), white floating particles were produced in the reaction mixture that readily disappeared upon the addition of EDTA. Therefore, the morphology of the liposomes was examined by cryo-electron microscopy. Herein, phospholipid biosynthesis was induced until PG formation. As expected, the liposomes added to the reaction mixture showed a general appearance as small unilamellar vesicles with sizes in the range of 30−100 nm (Figure 7a panel 1). Upon the addition of a large quantity of oleic acid, significant swelling and likely fusion of the liposomes was noted, showing an increase in size to 60−200 nm (Figure 7a panel 2), which confirms the notion of swelling as derived from the R18 assay. Next, the enzymes and their substrates were added and phospholipid biosynthesis was initiated until complete depletion of the FAs. This resulted in the formation of the white particles that by cryoEM are visible as stacked membrane layers comprising multilamellar like structures (Figure 7a panel 3). Addition of EDTA resulted in a complete dissolution of the stacked membranes and dissolved the white floating particles (Figure 7a panel 4) yielding normal liposomes. EDTA addition did not affect the final R18 fluorescence signal (data not shown).

The phenomenon of white particles already occurs with the production of PA, but was not noted during synthesis of LPA. As the stacked layers appear in time, likely during PG synthesis

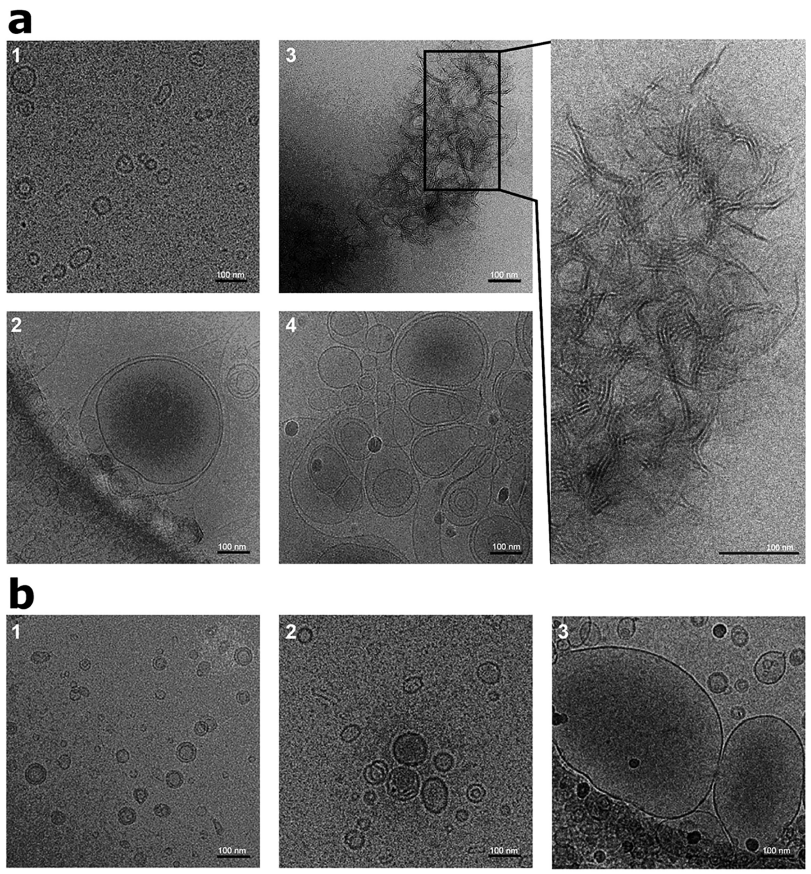

Figure 7. Transmission electron microscopic imaging of *in vitro* synthesized membranes. (a) Different phases during phospholipid biosynthesis, starting with small unilamellar liposomes (a1), the addition of 1.5 mM oleic acid to yield large swollen vesicles (a2), conversion of oleic acid into phospholipid resulting in stacked bilayer formation (a3), which is reversed upon addition of EDTA (a4) to yield large liposomes. (b) Different phases during phospholipid biosynthesis upon the dosed addition of small amounts of oleic acid. Starting with small unilamellar vesicles (b1), first addition of 0.3 mM oleic acid which does not result in observable vesicle swelling (a2), and formation of large vesicles, after multiple additions of small amounts of oleic acid to a final concentration of 1.5 mM, converted into phospholipid (b3).

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Table 2. Cloning and Expression Vectors Used in This Study

plasmid	description	reference
pRSF-Duet-1	Expression vector (Kan^R), T7 promoter	Novagen
pET-Duet-1	Expression vector (Amp^R), T7 promoter	Novagen
pET-28b	Expression vector (Kan^R), T7 promoter	Novagen
pACYC-Duet-1	Expression vector (Cm^R), T7 promoter	Novagen
pME001	*fadD* gene with C-terminus His-tag from *E. coli* MG1655 cloned into pRSF-Duet-1 vector using the primers PrME001 and PrME002	This study
pME002	*plsB* gene with C-terminus His-tag from *E. coli* MG1655 cloned into pet-28b vector using the primers PrME003 and PrME004	This study
pME003	*plsC* gene with C-terminus His-tag from *E. coli* MG1655 cloned into pet-28b vector using the primers PrME005 and PrME006	This study
pSJ148	*cdsA* gene with N-terminus His-tag from *E. E. coli* MG1655 cloned into pACYC-Duet vector using the primers 103 and 106	Caforio *et al.*[a]
pAC004	*pss* gene with C-terminus His-tag from *B. subtilis* cloned into pACYC-Duet vector using the primers 89 and 90	Caforio *et al.*[a]
pAC008	*psd* gene with C-terminus His-tag from *E. coli* MG1655 cloned into pACYC-Duet vector using the primers 533 and 534	Caforio *et al.*[a]
pAC015	*pgsA* gene with C-terminus His-tag from *E. coli* MG1655 cloned into pRSF-Duet vector using the primers 551 and 552	Caforio *et al.*[a]
pAC017	*pgpA gene* with C-terminus His-tag from *E. coli* MG1655 cloned into pET-Duet vector using the primers 562 and 563	Caforio *et al.*[a]

[a]Ref 24.

Table 3. Oligonucleotide Primers Used in This Study

primers	primer sequence 5′ → 3′	restriction site
PrME001	TACTAGGAATTCATGAAGAAGGTTTGGCTTAACCG	*Eco*RI
PrME002	AGTCATGCGGCCGCTCAGTGGTGGTGGTGGTGGTGGGCTTTATTGTCCACTTTGC	NotI
PrME003	CATCCTGCCCATGGCCGGCTGGCCACGAATTTACTAC	NcoI
PrME004	CTTCATGATTCTCGAGCCCTTCGCCCTGCGTCGCAC	XhoI
PrME005	CACTACGACCATGG TATATATCTTTCGTCTTATTATTACCG	NcoI
PrME006	CATGTCTACTCGAGAACTTTTCCGGCGGCTTC	XhoI
89	GCGCCATATGAATTACATCCCCTGTATGATTACG	NdeI
90	GCGCCTCGAGTTAGTGATGGTGATGGTGGTGATGATGATTCCATCTCCCAGACTCCAG	XhoI
103	GCGCCTCGAGTTAGTGATGGTGATGGTGGTGATGATGAAGCGTCCTGAATACCAGTAAC	XhoI
106	GCCGCCATGGGCAGCCATCACCATCATCACCACAGCCTGAAGTATCGCCTGATATCTGC	NcoI
533	GCGCCTCGAGAAAACAATGGCCTGGAGGCTACCTTGTTAAATTCATTTAAACTTTCGCTAC	XhoI
534	GCGCTTAATTAATTAGTGATGGTGATGGTGGTGATGATGGACCTGGTCTTTTTTGTCGTCAAC	PacI
551	GCGCCATATGCAATTTAATATCCCTACGTTGC	NdeI
552	CGCGCTCGAGTCAGTGATGGTGATGGTGGTGATGATGCTGATCAAGCAAATCTGCACGC	XhoI
562	CGCGGAATTCATGACCATTTTGCCACGCCATAAAG	*Eco*RI
563	CGGCGCGGCCGCCTAGTGATGGTGATGGTGGTGATGATGCGACAGAATACCCAGCG	NotI

a critical concentration of the intermediate PA is reached, which induces the white precipitation (Figure S4). PA strongly interacts with divalent cations, such as Ca^{2+} and Mg^{2+} *via* its negative charge, which can even result in the loss of liposomal structures. In our system, this process may even be accelerated by uneven growth of the outer leaflet of the membrane as compared to the inner leaflet as the enzymes and substrates are added from outside of the liposomes. As spontaneous phospholipid flip-flop is a slow process (hours), while these lipids are synthesized in an asymmetric manner, we hypothesize that the formation of Ca^{2+}/Mg^{2+} complexes of PA cause membrane stacking leading to the tubular multilamellar structures seen by cryo-EM. These divalent cation-PA complexes likely are unable to undergo flip-flop movements. As a consequence of large quantities of PA together with an imbalanced growth of the outer membrane leaflet size, the vesicle-like structures are disrupted and stacked membranes are obtained. This idea is supported by our observation that addition of EDTA results in the formation of normal vesicle structures.

Since the white precipitation is an undesired phenomenon, reactions were repeated but now with a lower Mg^{2+} concentration to prevent or slow down stacked layer formation. Furthermore, the addition of oleic acid was dosed over time, which results in a more gradual formation of the PG, thereby keeping intermediate PA levels at a minimum. Again, the starting material are small unilamellar liposomes with sizes in the range of 30−100 nm (Figure 7b panel 1). Addition of the first small dose of oleic acid together with the enzymes and their substrates resulted in hardly any membrane expansion and no vesicle fusion (Figure 7b panel 2). However, after applying several doses of oleic acid, larger vesicles could be detected, indicating growth of the liposomes. Furthermore, no stacked layer formation could be observed (Figure 7b panel 3) which underscores the need to control membrane expansion by a dosed feed of FAs.

CONCLUSIONS

Here, we report the development of an efficient and versatile system to generate phospholipids from simple fatty acids, thereby growing membranes in a test tube. Membrane biosynthesis is an important and essential step in the development of synthetic cells. The system is based on a cascade of eight purified (membrane) proteins, which are reconstituted into pre-existing liposomes, allowing for the incorporation of diverse fatty acyl chains and variations in the polar headgroup composition to mimic the phospholipid composition of a biological membrane, while recycling the CoA for continuous phospholipid synthesis.

The membrane expansion obtained *via* this system is an important step forward toward self-growing vesicles, but further

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steps have to be taken. It should be noted that 2.7 mM of oleic acid in the reaction mixture was about the maximal tolerable concentration, as higher concentrations of oleic acid at the start of the experiment inhibited biosynthesis (data not shown). For continuous phospholipid biosynthesis and membrane growth, we therefore envision a controlled feeding system in which oleic acid and other FAs are continuously added to the reaction vessel, such not to exceed inhibitory concentrations and prevent the formation of stacked membranes. Taken together, our reconstituted system truly represents a growing cellular like compartment that can be used to increase the complexity and address cellular processes that are linked to membrane growth, such as membrane protein insertion, phospholipid flip-flop and cell division.

METHODS

Bacterial Strain and Cloning Procedures. Genomic DNA of *Escherichia coli* was used as a template for the amplification of genes encoding for the enzymes FadD, PlsB and PlsC. *E. coli* DH5α (Invitrogen) was used for cloning. Plasmids for the *B. subtilis* enzyme PssA and *E. coli* enzymes CdsA, PgsA, PgpA and Psd were as reported.[24] All primers and plasmids used in the present study are listed in Tables 2 and 3. *E. coli* BL21 (DE3) was used as a protein overexpression host strain and grown under aerobic conditions at 37 °C in LB medium supplemented with the required antibiotics, kanamycin (50 μg/mL), chloramphenicol (34 μg/mL) and ampicillin (50 μg/mL).

Expression and Purification of Phospholipid Synthesizing Enzymes. Proteins involved in phospholipid synthesis were overexpressed in *E. coli* BL21 strain and induced with 0.5 mM IPTG. After 2.5 h. of induction (4 h. for inducing PssA and PgsA synthesis), cytoplasmic and membrane fractions were separated as described.[38] FadD present in the cytoplasmic fraction (supernatant) was stored at −80. For all other proteins, the total membranes fractions were resuspended in buffer A (50 mM Tris/HCl, pH 8.0, 100 mM KCl and 15% glycerol) after which they could be stored at −80 as well. For further purification, 0.5 mg/mL of membranes were solubilized in 2% *n*-dodecyl-β-D-maltopyranoside (DDM) detergent for 1 h. at 4 °C. The material was subjected to a centrifugation (17 000*g*) step for 15 min at 4 °C to remove insolubilized material and the supernatant was incubated with Ni-NTA (Ni^{2+}- nitrilotriacetic acid) beads (Sigma) for 2 h. at 4 °C. The Ni-NTA beads were washed 10 times with 40 column volumes (CV) of buffer B (50 mM Tris/HCl, pH 8.0, 100 mM KCl, 15% glycerol and 0.05% DDM) supplemented with 10 mM imidazole, and the proteins were eluted three times with 0.5 CV of buffer B supplemented with 300 mM imidazole. The cytoplasmic fraction of FadD was further purified *via* Ni-NTA agarose affinity chromatography using the same method as described above, with the exception that buffer B did not contain any DDM. Purity of the eluted proteins were assessed on 12% SDS/PAGE stained with Coomassie Brilliant Blue and the protein concentration was determined by measuring the absorbance at 280 nm. Extinction coefficients were obtained from the ProtParam tool from ExPASy by applying the specific amino acid sequence.

Liposomes Preparation. Chloroform stocks of the lipid species DOPG, DOPE, DOPC or POPG, POPE, DOPC (Avanti Biochemicals, Birmingham, AL) were mixed together in a ratio of 33:33:33, unless stated otherwise. Next the lipid solution was dried under a nitrogen gas stream for multiple hours, after which the dry lipid film was resuspended in a 50 mM Tris/HCl, pH 8.0 buffer. For formation of liposomes, a sonication cycle of 15 s on, 15 s off was repeated for 10−40×.

***In Vitro* Assays for Phospholipid Production.** All *in vitro* reactions were performed in 100 μL of assay buffer A containing a final concentration of 50 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM KCl, 15% glycerol and 2 mM DTT. The activity of FadD was assayed in buffer A using 0.5 μM enzyme and 550 μM of oleic acid, 40 μM or 250 μM CoA and with or without 1 mM ATP. The influence of lipids on FadD activity was examined under similar conditions with addition of 2.67 mM of different lipids as indicated. Activity assays of PlsB and PlsC were performed in buffer A with addition of 0.1% DDM, 100 μM oleoyl-CoA, 0.5 μM enzyme and with or without 2.67 mM of various lipid mixtures, 10 mM G3P or 120 mM LPA (see figure legends). Combined activity assays of PlsB and PlsC were performed in buffer A with addition of 0.5% DDM, 100 μM oleoyl-CoA, 2.67 mM of phospholipid, 1 μM PlsB and 1.5 μM of PlsC and 10 mM G3P, unless indicated otherwise.

The stepwise cascade conversion of oleic acid into PA was assayed in buffer A in the presence of 0.5 μM FadD, 550 μM or (2.7 mM) Oleic acid, 50 μM CoA, 2.67 mM of liposomes, 0.5 μM PlsB and 1.5 μM PlsC, 1 (or 4) mM ATP and 10 mM G3P as indicated in the figure legends. The conversion of multiple fatty acids into a wide variety of PAs was assayed as above, but with 675 μM of palmitoleic acid, palmitic acid, oleic acid and stearic acid, respectively.

Conversion of oleic acid into PG and/or PE was assayed in buffer A with addition of 0.5 μM FadD, 50 μM CoA, 550 μM or 2700 μM oleic acid, 1 mM or 4 mM ATP, 2.67 mM liposomes, 10 mM G3P, 0.5 μM PlsB, 1.5 μM PlsC and, as indicated, 2 μM CdsA, 3 mM CTP, 1 μM PgsA, 1 μM PgpA, 1 μM PssA, 3 mM L-serine and 1 μM Psd, unless indicated otherwise.

All reactions were incubated overnight at 37 °C unless stated differently. Lipids were extracted two times with 0.3 mL of n-butanol, and evaporated under a stream of nitrogen gas and resuspended in 50 μL of methanol for LC−MS analysis.

Fluorescent Assays for Membrane Expansion. To visualize membrane expansion, Octadecyl Rhodamine B chloride (R18) (Biotium Inc. Fremont, USA) was incorporated into the liposomes at a concentration of 5 mol % during liposome preparation. Synthesis reactions were performed in buffer A with addition of 0.5 μM FadD, 50 μM CoA, 2700 μM oleic acid, 2.67 mM R18 liposomes (DOPC, DOPG, DOPE), 10 mM G3P, 0.5 μM PlsB, 1.5 μM PlsC and, if present 4 mM ATP and 1% Triton X-100. Reactions were quenched with 10 mM EDTA, and fluorescence was excited at 540 nm and emission spectra (560−680 nm) recorded using a QuantaMaster spectrofluorometer controlled by the FelixGX program (Photon Technology International, Inc.).

Cryotransmission Electron Microscopy. A few microliters of sample was deposited on a holey carbon coated copper grid (Quantifoil 3.5/1). After blotting the excess of the sample with filter paper, the grids were plunged quickly into liquid ethane (FEI vitrobot). Frozen-hydrated specimens were mounted in a cryo-stage (Gatan, model 626) and observed in a FEI Tecnai T20 electron microscope, operating at 200 kV. Micrographs were recorded under low-dose conditions on a slow-scan CCD camera (Gatan, model 794).

LC−MS Analysis of Lipids. Samples from the *in vitro* reactions were analyzed using an Accela1250 HPLC system coupled with an ESI−MS Orbitrap Exactive (Thermo Fisher

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Scientific) as described.[38] In short, 5 μL was injected into a COSMOSIL 5C4-AR-300 Packed Column, 4.6 mm I.D. × 150 mm (Nacalai USA, Inc.) operating at 40 °C with a flow rate of 500 μL/min. Separation of the compounds was achieved by a changing gradient of Mobile phase A (50 mM ammonium bicarbonate in water) and mobile phase B (Acetontrile). The MS settings and specifications used for this analysis were the same as described before.[38] Only for the simultaneous synthesis of PE and PG (Figure 5c) 5 μL of sample was injected into a Shim-pack XR-ODS/C18 column with dimension 3.0 mm × 75 mm (Shimadzu) operating at 55 °C with a flow rate of 400 μL/min. Separation of the compounds was achieved by a changing gradient of Mobile phase A (10 mM ammonium formate with 0.1% formic acid in water/acetonitrile 40:60, v/v) and mobile phase B (10 mM ammonium formate with 0.1% formic acid in acetonitrile/propan-2-ol, 10:90, v/v).[24] The MS settings and specifications used for this analysis were the same as described before.[24]

Spectral data constituting total ion counts were analyzed using the Thermo Scientific XCalibur processing software by applying the Genesis algorithm based automated peak area detection and integration. The total ion counts of the extracted lipid products: oleic acid (m/z 281.25 $[M - H]^-$), oleoyl-CoA (m/z 1030.35 $[M - H]^-$), LPA (m/z 435.26 $[M - H]^-$), (DO)PA (m/z 699.49 $[M - H]^-$), CDP-DAG (m/z 1004.54 $[M - H]^-$), PGP (m/z 853.50 $[M - H]^-$), DOPG (m/z 773.53 $[M - H]^-$), PS (m/z 786.53 $[M - H]^-$), DOPE (m/z 742.54 $[M - H]^-$), palmitoleic acid (C16:1) (m/z 253.22 $[M - H]^-$), palmitic acid (C16:0) (m/z 255.23 $[M - H]^-$), oleic acid (C18:1) (m/z 281.25 $[M - H]^-$), stearic acid (C18:0) (m/z 283.26 $[M - H]^-$). LPA (C16:1) (m/z 407.22 $[M - H]^-$), LPA (16:0) (m/z 409.23 $[M - H]^-$), LPA (C18:1) (m/z 435.25 $[M - H]^-$), LPA (C18:0) (m/z 437.26 $[M - H]^-$). PA (C16:1−16:1) (m/z 643.43 $[M - H]^-$), PA (16:0−16:0) (m/z 647.46 $[M - H]^-$), PA (C18:1−C16:1) (m/z 671.46 $[M - H]^-$), PA (C16:1−16:0) (m/z 645.45 $[M - H]^-$), PA (C18:0−16:1/18:1−16:0) (m/z 673.48 $[M - H]^-$), PA (18:1−18:0) (m/z 701.51 $[M - H]^-$), PA (C18:0−C16:0) (m/z 675.49 $[M - H]^-$), PA (C18:1−18:1) (m/z 699.49 $[M - H]^-$), PA (18:0−18:0) (m/z 703.52 $[M - H]^-$) were normalized for either DDM (m/z 509.3 $[M - H]^-$) or POPG (m/z 747.52 $[M - H]^-$) and plotted on the y-axis as normalized ion count.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00265.

Figures S1−S4; Supplemental experimental procedures (PDF)

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Author Contributions

Experiments were designed by M.E. with help of A.C. and A.J.M.D. Experiments were performed by M.E. with help of A.C. Electron microscopy related work was designed by M.C.A.S., M.E. and A.J.M.D. Electron microscopy was executed by M.C.A.S. and analyzed together with M.E. Manuscript was written by M.E. with improvements of A.J.M.D. Cloning of the enzymes CdsA, PgsA, PgpA, PssA and Psd was performed by A.C.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ATP, adenosine triphosphate; CDP-DAG, CDP diacylglycerol; CdsA, CDP-DAG synthetase; CMC, critical micelle concentration; CoA, Coenzyme A; CTP, cytidine triphosphate; DDM, *n*-dodecyl-β-D-maltopyranoside; DOPC, Dioleoylphosphatidylcholine; DOPE, Dioleoylphosphatidylethanolamine; DOPG, Dioleoylphosphatidylglycerol; FadD, Long-chain fatty acid-CoA ligase; FAs, Fatty acids; PA, phosphatidic acid; PlsB, glycerol-3-phosphate acyltransferase; PlsC, 1-acyl-*sn*-glycerol-3-phosphate acyltransferase; PgsA, Phosphatidylglycerophosphate synthase A; PgpA, Phosphatidylglycerophosphatase A; Psd, Phosphatidylserine decarboxylase; PssA, Phosphatidylserine synthase A

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